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Role for a Phage Promoter in Shiga Toxin 2 Expression from a Pathogenic *Escherichia coli* Strain

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Shiga toxins (Stxs), encoded by the *stxA* and *stxB* genes, are important contributors to the virulence of *Escherichia coli* O157:H7 and other Stx-producing *E. coli* (STEC) strains. The *stxA* and *stxB* genes in STEC strains are located on the genomes of resident prophages of the λ family immediately downstream of the phage late promoters ($p_{R'}$). The phage-encoded Q proteins modify RNA polymerase initiating transcription at the cognate $p_{R'}$ promoter which creates transcription complexes that transcend a transcription terminator immediately downstream of $p_{R'}$ as well as terminator kilobases distal to $p_{R'}$. To test if this Q-directed processive transcription plays a role in stx_2AB expression, we constructed a mutant prophage in an O157:H7 clinical isolate from which $p_{R'}$ and part of Q were deleted but which has an intact *p*Stx, the previously described stx_2AB-associated promoter. We report that production of significant levels of Stx2 in this O157:H7 isolate depends on the $p_{R'}$ promoter. Since transcription initiating at $p_{R'}$ ultimately requires activation of the phage lytic cascade, expression of stx_2AB in STEC depends primarily on prophage induction. By showing this central role for the prophage in stx_2AB expression, our findings contradict the prevailing assumption that phages serve merely as agents for virulence gene transfer.

Escherichia coli O157:H7 and other Shiga toxin (Stx)-producing *E. coli* (STEC) strains are responsible for outbreaks and sporadic cases of diarrhea. In some patients, exposure to STEC leads to hemorrhagic colitis and hemolytic uremic syndrome that may lead to death (20). Two major classes of Stxs, Stx1 and Stx2, encoded respectively by stx_1AB and stx_2AB, have been identified in STEC (2). The severe clinical consequences of STEC infections are thought to be caused by the activities of Stxs, although Stx2 appears to be more closely associated with these sequelae than does Stx1 (7, 28, 36). Shiga toxins are of the A-B type, with the glycolipid-binding B subunits being involved in the transport of the enzymatic A subunits into the eukaryotic cell where the A subunit, acting as a glycosylase, catalyzes a cleavage at a unique site in the 28S rRNA (2). The resulting inactivation of the ribosome leads to an inhibition of protein synthesis. More than 60 serotypes of STEC have been associated with human disease (1). The *stx* genes of many, if not all, STEC strains are in the genomes of prophages of the lambdoid family (19, 23, 27). This fact probably accounts for the wide dissemination of these genes in diverse *E. coli* serotypes.

Comparison of lambdoid phage genomes (9) has revealed a common arrangement of functionally similar genes and a shared strategy governing gene expression (Fig. 1). In the lysogenic state, the repressor silences transcription of most phage genes (30). Removal of repression, which can occur when DNA damage activates the bacterial SOS response causing RecA-mediated cleavage of the repressor (21), leads to a cascade of regulatory events beginning with expression of the N transcription antitermination protein (30). Terminator readthrough mediated by the N protein results in expression of delayed early genes that encode products involved in replication and prophage excision, as well as the Q antitermination protein (15). Q acts at a site, *qut*, within the phage late promoter, $p_{R'}$, modifying RNA polymerase to a highly processive form that reads through downstream terminators (39), including the strong Rho-independent terminator, $t_{R'}$, located directly downstream of $p_{R'}$ (32, 33). Thus, late gene expression by lambdoid prophages is a consequence of prophage induction; i.e., it follows removal of repression of the early promoters, p_R and p_L.

Although previous studies have identified functional promoters immediately upstream of *stx* genes (8, 34), recent evidence suggests that prophage induction and the resulting transcription from the phage $p_{R'}$ late promoters are likely to be important in *stx* expression (26). First, *stx* genes are located directly downstream of the $p_{R'}$ promoters and upstream of the phage lysis genes (25, 26, 29). Second, agents that induce Stx phages also increase Stx expression by their host STEC strains (24, 40). Third, the Q protein from Stx phage H-19B acting in *trans* directs high-level expression from the *stx* genes of repressed H-19B and 933W prophages, two phages that share nearly identical Q, *qut*, and $p_{R'}$ sequences but different *stx* genes (25, 29). Thus, these observations suggest that the regulatory circuits of Stx-encoding phages play a direct role in STEC pathogenesis. Moreover, if phage-directed lysis is important in Stx release from STEC cells, Q-activated transcrip-

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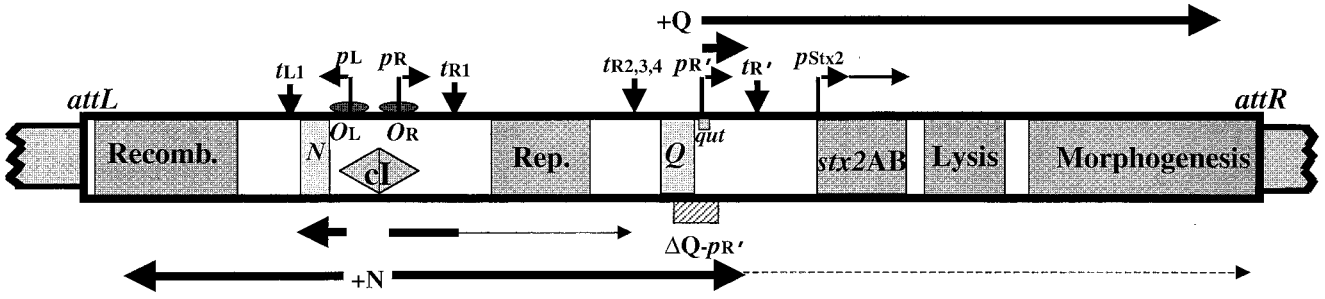


FIG. 1. Presumed genome arrangement and transcription patterns of Stx2-encoding phage Φ361. This diagram is based on the maps of characterized lambdoid phages (16, 26), including the Stx2-encoding phage 933W (29), and the information we have on Φ361 (not drawn to scale). Shown are relevant genes, promoters, terminators, operators, and the site of the ΔQ-$p_{R'}$ deletion. The *attL* and *attR* sites are the junctions of the integrated prophage with the bacterial DNA. Below are shown the patterns of transcription initiating at the early promoters, p_L and p_R, in the absence and presence of N. Above are shown the patterns of transcription initiating at the late promoter $p_{R'}$ in the absence and presence of Q. Induction inactivates the repressor (*c*I), resulting in expression of the N protein-encoded antiterminator. N modification of RNA polymerase allows read-through of the t_{L1} and t_{R1} terminators, resulting in production of proteins catalyzing excision and replication of the phage genome. The N-modified RNA polymerase also reads through the $t_{R2, -3}$, and $_{-4}$ terminators, leading to synthesis of the Q antiterminator. The Q-modified RNA polymerase transcends transcription termination at $t_{R'}$ and subsequent downstream terminators, allowing expression of late genes, which include those encoding proteins involved in lysis and morphogenesis. Recomb., recombination; Rep., replication.

tion of *stx* and downstream lysis genes may serve as the primary mechanism for coordinating production and release of toxin during a STEC infection (26, 38). We present evidence that transcription from the late phage promoter $p_{R'}$ resulting from prophage induction plays a major role in the production of toxin from the Stx2-encoding O157:H7 enterohemorrhagic *E. coli* clinical isolate 1:361.

MATERIALS AND METHODS

Bacteria and plasmids. Strain 1:361, a clinical *E. coli* isolate of serotype O157:H7, has previously been described (37). Strain 1:361ΔQ-$p_{R'}$ was derived from 1:361 using the allele exchange vector pΔQ-$p_{R'}$. This plasmid is a derivative of the *sacB* counterselectable vector pCVD442 (13), which contains an ~900-bp DNA fragment from the $p_{R'}$ region of Φ361 with a 189-bp deletion that includes $p_{R'}$. The inserted fragment was synthesized using the procedure involving PCR-based splicing by overlap extension (18) with the 1:361 chromosomal DNA serving as the template. Four primers were employed in constructing this fragment: the upstream primer 5′CACCGTAAAAACCATTCCTGACATGCTCC, corresponding to sequences in the Q gene; the overlapping primers 5′CCTTTC TGTGTACTTTCCGCCAGCATCATCAGCATGCC and 5′GGCATGCTGAT GATGCTGGCGGAAAGTACACAGAAAGG, corresponding to sequences upstream and downstream of the deleted region; and 5′GCCACCACATTAAC TGAAAAGATAAC, corresponding to sequences downstream of $p_{R'}$. pΔQ-$p_{R'}$ was introduced into 1:361 by conjugation from *E. coli* strain SM10λpir. Exconjugates were selected as streptomycin- and ampicillin-resistant colonies. Haploid cells were then selected as sucrose-resistant colonies as described previously (13) and subsequently screened for ΔQ-$p_{R'}$ mutations. DNA sequence analysis confirmed that 1:361ΔQ-$p_{R'}$ contains the proper deletion.

RNA preparation. RNA from mid-log-phase culture of 1:361 or 1:361ΔQ-$p_{R'}$ was prepared using an RNeasy kit (Qiagen). Northern blotting was performed using standard procedures (3) with a ^{32}P-labeled stx_2A riboprobe. The stx_2A probe was synthesized using T7 polymerase (Ambion), with plasmid pPW58 (which contains 258 bp of stx_2A coding sequence subcloned into plasmid pCR2.1-TOPO [Invitrogen]) as the template.

Animal model. A modified version of the streptomycin-treated-mouse model of enterohemorrhagic *E. coli* infection (35) was used to compare the levels of colonization and intestinal Stx2 production of 1:361 and 1:361ΔQ-$p_{R'}$. Four-week-old CD-1 mice were given drinking water containing streptomycin (2 g/liter) throughout each experiment. Two days after streptomycin treatment was begun, mice were inoculated intragastrically with ~10^{10} CFU of either 1:361 or 1:361ΔQ-$p_{R'}$. Fecal samples were collected daily beginning 1 day after inoculation. Numbers of streptomycin-resistant CFU of either 1:361 or 1:361ΔQ-$p_{R'}$ and fecal Stx2 concentration were determined as described previously (41).

Toxin assay. Stx levels were measured using an enzyme-linked immunosorbent assay (ELISA) essentially as previously described (14).

RESULTS

Prophage construction. *E. coli* strain 1:361, isolated from a patient with bloody diarrhea, contains two Stx2-encoding lambdoid prophages (data not shown). One of these phages was found to be defective, and the other, designated Φ361, shares, at a minimum, the *Q*, $p_{R'}$, and stx_2 sequences found in phage 933W (37) (Fig. 1). We constructed a derivative of 1:361, called 1:361ΔQ-$p_{R'}$, in which a 189-bp region including the 3′ end of *Q* and the $p_{R'}$ late promoter of the Φ361 prophage was deleted (Fig. 1). This deletion left intact the stx_2A and *-B* genes and the previously identified stx_2 promoter, *p*Stx2. As expected for a lysogen carrying a lambdoid prophage defective in transcription from $p_{R'}$, 1:361ΔQ-$p_{R'}$ did not produce phage detectable by plaque formation following induction with mitomycin C (Fig. 2C).

Transcription levels. Northern blotting was performed to directly assess the role of transcription initiating at $p_{R'}$ in *stx* expression (Fig. 2A). Strain 1:361 produced low levels of the *stx* transcript (lane 1), while strain 1:361ΔQ-$p_{R'}$ (lane 3) failed to produce detectable levels of the *stx* transcript. Following induction with mitomycin C, strain 1:361 produced high levels of the *stx* transcript (lane 2), while strain 1:361ΔQ-$p_{R'}$ again failed to produce observable levels of the *stx* transcript (lane 4). Based on studies with λ (33), Q-mediated antitermination of transcription initiating at $p_{R'}$ is expected to result in a >25-kb message; however, the relatively small size of the *stx* transcript is not surprising for an mRNA initiating at $p_{R'}$ since the λ $p_{R'}$ message is known to be processed (11). Therefore, these experiments provide evidence that the Q-$p_{R'}$ region in Φ361, which is necessary for transcription of late phage genes, is also important for stx_2 transcription.

Toxin levels. Stx levels in cultures of 1:361 and 1:361ΔQ-$p_{R'}$, measured by an ELISA (14), provide further evidence that transcription from $p_{R'}$ contributes significantly to *stx* expression (Fig. 2B). Uninduced cultures of strain 1:361 produced low levels of Stx2, and under identical conditions, cultures of 1:361ΔQ-$p_{R'}$ failed to produce measurable levels of Stx2. Thus, the low level of Stx produced by the untreated culture most

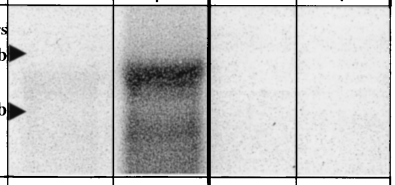
Treatment/ Product Measured	Bacteria			
	1:361		ΔQ-pR'	
	1	2	3	4
Mitomycin C	-	+	-	+
A RNA Northern Blot				
B Stx2 (ng/ml)	9.0 +/- 1.4	167.3 +/-18.0	<0.1	9.2 +/- 0.8
C Phage Titer (pfu/ml)	2.1 x 10^4	2.0 x 10^9	0	2 x 10^1

FIG. 2. Production of stx_2 RNA, Stx2, and phage from STEC strains 1:361 and 1:361ΔQ-$p_{R'}$. (A) Measurement by Northern blotting of *stx* mRNA levels. Total RNA was prepared from 3-h cultures of 1:361 and 1:361ΔQ-$p_{R'}$ grown with (+) or without (−) 0.5 μg of mitomycin C per ml. Six micrograms of RNA was run on a 1% agarose gel, transferred to a nylon membrane, and then probed with a ^{32}P-labeled Stx2-encoding sequence riboprobe. (B) Total Stx2 was measured from the same cultures used in the Northern blot, using a previously described ELISA (14). Values with standard deviations from three independent measurements are shown. (C) Phage titers were determined using *E. coli* C600 as the indicator strain (3). This procedure leaves uncounted mutant phage producing very low bursts.

likely resulted from Q-stimulated transcription from $p_{R'}$ in the small fraction of lysogens in which there was spontaneous induction of the prophage. Induced cultures of 1:361 (treated with mitomycin C) produced high levels of Stx2, while similarly treated cultures of 1:361ΔQ-$p_{R'}$ produced toxin levels that were nearly 20-fold less (Fig. 2B). These results confirm the conclusion from the Northern analysis that the Q-$p_{R'}$ region exerts a critical role in regulating Stx2 synthesis in vitro.

Stx production in vivo. To assess the physiological relevance of these findings, Stx2 production by strains 1:361 and 1:361ΔQ-$p_{R'}$ was examined in vivo, using a mouse model system (35). In these experiments, CD-1 mice were inoculated intragastrically with either 1:361 or 1:361ΔQ-$p_{R'}$. Over the course of 4 days, stool samples were collected and assayed both for CFU of 1:361 or 1:361ΔQ-$p_{R'}$ and for levels of Stx2. In striking contrast to the similar numbers of 1:361 and 1:361ΔQ-$p_{R'}$ CFU recovered, the difference in the amounts of Stx in the stool specimens between the mice inoculated with the two strains was dramatic (Fig. 3). There was an approximately 30-fold lower concentration of Stx2 in stools from animals inoculated with 1:361ΔQ-$p_{R'}$ than the concentration of Stx2 in stools from mice inoculated with 1:361. Thus, Stx production by strain 1:361 in the animal gut, as in vitro culture, largely derives from the small fraction of the bacteria in which there is prophage induction.

DISCUSSION

Previous studies identified a functional promoter, *p*Stx, immediately upstream of *stx* genes in the Stx1-encoding phage H-19B (8) and the Stx2-encoding phage 933W (34). These findings appeared to confirm the idea that, although *stx* genes can be carried by phages, once the phage is established as a prophage, *stx* is expressed independently of the regulatory system governing phage gene expression. More recent studies led to the suggestion that phage-encoded regulatory circuits that result in Q modification of transcription initiating at the phage $p_{R'}$ promoter and subsequent processive transcription of downstream genes, including *stxA* and *stxB*, may play a role in *stxAB* expression (26). We directly tested this hypothesis by deleting the Q-$p_{R'}$ region while leaving intact the *p*Stx promoter of a prophage that carries the stx_2A and *-B* genes in an *E. coli* O157:H7 clinical isolate.

Our experiments comparing levels of Stx2 production by the parent clinical strain and an isogenic derivative with a deletion of the Q-$p_{R'}$ region of the prophage provide compelling evidence supporting the idea that Q-activated $p_{R'}$-promoted processive transcription plays a critical role in Stx2 production. Since Q expression depends on transcription from p_R, a promoter that is under repressor control, *stx* expression, according to this idea, ultimately depends on induction of the prophage. Induction is expected to result in increased *stx* copy number and increased transcription of phage late genes initiating from the early promoter p_R. However, copy number and increased transcription of phage late genes by N-antiterminated transcription from the early promoter p_R are not expected to be affected by the ΔQ-$p_{R'}$ deletion (17). Therefore, our results strongly suggest that high-level Q-modified transcription initiating at the Φ361 $p_{R'}$ late promoter is responsible for the increase in Stx2 production observed upon induction of strain 1:361. Residual toxin production by 1:361ΔQ-$p_{R'}$ may be due

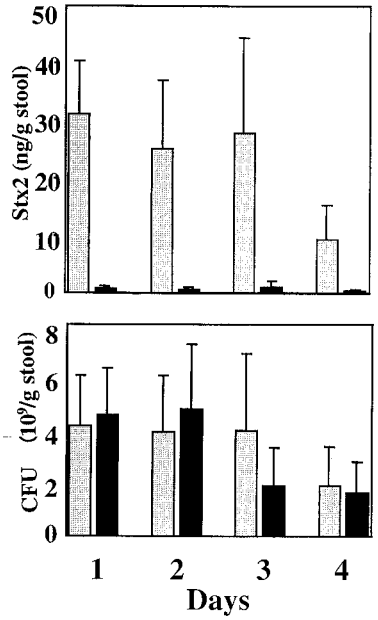


FIG. 3. Fecal cell counts of 1:361 and 1:361ΔQ-$p_{R'}$ and Stx2 concentration. Streptomycin-treated CD-1 mice were intragastrically inoculated with either 1:361 or 1:361ΔQ-$p_{R'}$. Stool samples were collected daily, and the Stx2 concentration (top) and the number of CFU (bottom) of either 1:361 or 1:361ΔQ-$p_{R'}$ were determined. Gray bars represent mice inoculated with 1:361, and black bars represent mice inoculated with 1:361ΔQ-$p_{R'}$. There were eight mice in each group. Means and standard deviations are presented.

to low-level transcription from the early $p_{R'}$ promoter (10) or *p*Stx2 and possibly from the other defective Stx2-encoding phage present in 1:361. Our results are consistent with the hypothesis that Q-modified transcription (32) beginning at $p_{R'}$ proceeds through the terminator $t_{R'}$, into the *stx* genes, past termination signals, and through the downstream lysis genes, providing a unified mechanism for coupling Stx production and release. Previously, Stx phages were considered to be important in the dissemination of but not the regulation of expression of toxin genes (38). Our results favor the view that stx_2 expression is regulated as one of the late phage genes and suggest that, far from being a mere vector for toxin gene transfer, Φ361 directs production of Stx2 as part of its lytic cycle. If our model is correct, it would mean that an induced subpopulation of the total infecting STEC population is responsible for significant levels of Stx production. This does not appear to be the exclusive mode for regulating virulence gene expression in lambdoid phages. The prototypical λ itself encodes *bor* and *lom*, genes encoding proteins having significant homology to known bacterial virulence factors (5) and, in the case of *bor*, shown to confer a virulence phenotype (6). The biologically relevant expression of these genes, unlike that of the *stx* genes, occurs from the repressed prophage.

Our study raises a question about the evolution of *stx*-carrying phages. Since the subpopulation of bacteria postulated to be the primary producers of Stx would not survive to be infectious, how can evolution select for *stx* if its production is coupled with phage-mediated lysis? The diarrhea induced by Stx likely contributes to the dissemination of the remaining population, an effect that may outweigh selection against toxin producers. There is precedence for the idea that the death of a minority of a bacterial population contributes to the survival advantage of the majority. Bacteriocins, molecules produced by some bacteria that are lethal to other bacteria of the same and closely related species, are produced by a subpopulation that is lysed in the process of releasing the bacteriocin (31). A possible role for the phage in expression of phage-borne virulence genes has been considered since the early demonstration that diphtheria toxin is carried by a phage (4). As far as we know, the results of our study provide the first evidence directly linking a phage regulatory cascade with expression of a phage-encoded virulence factor.

Expression of virulence factors, even when carried by mobile genetic elements, is generally considered in the context of overall coordination of the infectious strategy of the host bacterium (22). Here we present evidence that the bacteriophage life cycle is the dominant, if not the only, important factor involved in Stx2 expression. It will be interesting to learn whether phage control of virulence gene expression is unique to *stx* or can serve as a paradigm that explains regulation of expression of other phage-encoded virulence factors. In light of our findings, an understanding of the conditions in the intestine that trigger prophage induction and subsequent upregulation of Stx expression takes on new significance. Notable pharmacologic agents which induce prophages include mitomycin C, used in chemotherapy, and numerous antibiotics, including the fluoroquinolones, often used in treatment of diarrhea (41). Interestingly, endogenous products of inflammatory cells, such as H_2O_2, are also known to induce lambdoid prophages (12). While it remains unknown to what extent such exogenous and endogenous factors contribute to STEC pathogenesis, our study suggests that clinical intervention to prevent prophage induction may reduce Stx production during STEC infection.

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P.L.W. and M.N.N. contributed equally to this work.

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